16S rRNA Gene Sequencing and Microbiome Analysis

Raw 16S rRNA gene sequencing data were processed using the **DADA2** pipeline (v1.30) in **R**. Primer sequences and low-quality regions were removed by truncating forward reads at 250 bp, and reverse reads at 160 bp, and trimming 20 bases from the 5′ end. Reads exceeding a maximum expected error threshold of 2 (maxEE = 2) were discarded. Following quality filtering, reads were dereplicated, and chimeric sequences were removed. Amplicon sequence variants (ASVs) were inferred and taxonomically classified using the **SILVA** reference database (release 138.2).

ASVs were aligned using **MAFFT** (v7.149), and a phylogenetic tree was constructed with **FastTree** (v2.1). The resulting ASV table and associated metadata were integrated into a **phyloseq** object (v1.46.0). Non-bacterial sequences and those annotated as Chloroplast or Mitochondria were removed. ASVs present in fewer than 25% of samples were also excluded to reduce sparsity.

To account for differences in sequencing depth, the ASV table was rarefied to an even sampling depth of 12,487 reads per sample. Relative abundances were computed by normalizing ASV counts to the total reads per sample.

For microbiome community composition analysis, the relative abundance profiles of microbial families in Fecal Buffer Blanks at time 0 (FBB0) and 16 hours (FBB16) were visualized using **ggplot2** (v3.5.2). Microbiome alpha diversity analysis was conducted for each individual microbiome by four different methods(Shannon, Chao 1, Simpson, PD). Community dissimilarity was assessed using four distance metrics: Bary-Curtis, Jaccard, Unweighted UniFrac, and Weighted UniFrac. Principal coordinate analysis (PCoA) was performed for each distance method, and the first two components were used to compute centroid positions for each category within a given microbiome. FBB0 coordinates served as reference starting points in visualizations depicting the dissimilarity trajectories across conditions. All visualizations were produced using **ggplot2** (v3.5.2).

Permutational Multivariate Analysis of Variance (PERMANOVA) based on Bray-Curtis distance matrices was used to assess genus-level compositional differences across sample groups. P-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate. Genera exhibiting significant variation between groups (adjusted *p* < 0.05) were retained for downstream analysis. The relative abundances of these significant taxa were log₁₀-transformed, and fold changes were visualized using heatmaps, stratified by treatment group and individual microbiome.

Based on the classification criteria described by Korth et al. (2024) [<https://doi.org/10.1093/g3journal/jkae145>], microbial taxa were categorized as either health-promoting or potentially harmful. These classifications were then used to calculate the Prebiotic Potential Index adjusted (PPIa), a composite metric reflecting overall gut microbiome health. Additionally, the Gut Microbiome Health Index (GMHI) was computed at each sample level following the method proposed by Gupta et al. (2020) [https://doi.or g/10.1038/s41467-020-18476-8].